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# Cultivation Conditions and Selenium Fertilization Alter the Phenolic Profile, Glucosinolate, and Sulforaphane Content of Broccoli

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ABSTRACT Broccoli is a food often consumed for its potential health-promoting properties. The health benefits of broccoli are partly associated with secondary plant compounds that have bioactivity; glucosinolates and phenolic acids are two of the most abundant and important in broccoli. In an effort to determine how variety, stress, and production conditions affect the production of these bioactive components broccoli was grown in the greenhouse with and without selenium (Se) fertilization, and in the field under conventional or organic farming procedures and with or without water stress. High-performance liquid chromatography/mass spectrometry was used to separate and identify 12 primary phenolic compounds. Variety had a major effect: There was a preponderance of flavonoids in the Majestic variety, but hydroxycinnamic esters were relatively more abundant in the Legacy variety. Organic farming and water stress decreased the overall production of phenolics. Se fertilization increased glucosinolates in general, and sulforaphane in particular, up to a point; above that Se fertilization decreased glucosinolate production. Organic farming and water stress also decreased glucosinolate production. These data show environmental and genetic variation in phenolics and glucosinolates in broccoli, and warn that not all broccoli may contain all health-promoting bioactive components. They further show that selection for one bioactive component (Se) may decrease the content of other bioactive components such as phenolics and glucosinolates.

KEY WORDS: • *broccoli* • *glucosinolates* • *high-performance liquid chromatography/ultraviolet/mass spectrometry* • *hydroxycinnamic acids* • *phenolics* • *selenium*

## INTRODUCTION

Functional foods, *i.e.*, foods that provide specific health benefits beyond basic nutrition, are one of the fastest growing segments of the food industry.[1] Many functional properties of foods are based on the actions of a specific bioactive component; phytochemicals in plant foods are especially important. However, many factors may affect the production and accumulation of bioactive compounds, and if a food is to be marketed based on a functional characteristic there must be a measure of the inherent variability.

Broccoli, one of the most commonly consumed vegetables in North America, is an excellent source of folate.[2] In an Australian study, broccoli was among the greatest contributors to lutein and zeaxanthin intakes.[3] Broccoli also is a primary food source of phylloquinone,[4] vitamin C,[5] and dietary fiber.[6]

While the above nutrients in broccoli are important, other non-nutritive components may be equally important for health. Glucosinolates, parent compounds of potent Phase II enzyme inducers such as sulforaphane (SF), may be responsible for the cancer-protective qualities of broccoli.[7] Another class of bioactive compounds found in all plant-based foods is phenolics. Chu *et al.*[8] reported broccoli to have the highest total phenolic content of the 10 most commonly consumed vegetables in the United States. Broccoli also has the unique ability to accumulate large amounts of selenium (Se) if grown under conditions where soluble Se is available. The form of Se in broccoli may be especially efficacious for the prevention of several cancers in biological systems.[9]

Phenolics are secondary metabolites of higher plants synthesized for a variety of roles in plant life (including herbivory defense and cell-to-cell signaling, thus increasing plant competitiveness).[10–12] Phenolics are nutritionally important because of their role as antioxidants, and in the inhibition of oxidative damage diseases such as coronary heart

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disease, stroke, and cancers.[13,14] Phenolics have been reported to suppress the formation of mutagenic compounds from salted fish,[15] and some authors have suggested that phenolics may explain some of the anti-cancer benefits of foods such as whole grains,[16] olive oil,[17] high-fiber foods (degradation of non–lignin-containing plant cell walls releases ferulic and other hydroxycinnamic acids),[18] citrus fruits,[19] and garlic.[20] Analysis of phenolic components is complicated by the large variety of natural derivatives (estimated to be near 8,000),[21] and because they are minor components in a complex plant matrix. In addition, both genetic (*e.g.*, cultivar) and environmental (*e.g.*, climate, soil type, and production practice) conditions may affect the phenolic profile and total content of a plant.

Glucosinolates are secondary plant compounds comprising a $\beta$-D-thioglucose group, a sulfonated oxime group, and an amino acid-derivative side chain.[22] More than 120 glucosinolates have been characterized, and although no essential role for glucosinolates in plant metabolism has been found, they may be important in herbivore and microbial defense.[22] Glucosinolates are not bioactive until they have been enzymatically hydrolyzed[23] by endogenous myrosinase, released during the disruption of the plant cell by harvesting, processing, or mastication. Hydrolysis of glucosinolates may also occur by gut microflora.[23] Broccoli converts 80–90% of glucoraphanin to SF nitrile and 10–20% to SF.[24] SF is the most potent Phase II inducer in broccoli, while SF nitrile has no effect on phase II enzyme activities. SF and other dietary isothiocyanates are of importance because they regulate phase I and II enzyme activities and thus may account for the cancer-protective effects of cruciferous vegetable. Glucosinolate concentrations are affected by environmental conditions including cultivation systems, climate, and soil conditions.[25]

In the present investigation, we report some effects of environment on the profile and accumulation of phenolics and glucosinolates in broccoli. Because broccoli accumulates Se that has accumulated onto soils after irrigation with Se-laden water,[26] which is used as a method of phytoremediation on high-Se soils, we have studied broccoli fertilized with Se, and show that exposure of broccoli to Se changes the phenolic profile and glucosinolate content. Also, production and consumption of high-cash-value organic vegetables, *e.g.*, broccoli have drastically increased during the last decades, so we wanted to test if organic farming practices altered the phenolic and glucosinolate profiles compared with conventionally grown broccoli in the same area.

## MATERIALS AND METHODS

### *Reagents*

Acetonitrile, formic acid, ethyl acetate, methanol, acetic acid, and HCl were all of analytical or high-performance liquid chromatography (HPLC) grade and purchased from Fisher (Fairlawn, NJ). All phenolic acids standards (ferulic, caffeic, sinapic, and chlorogenic) as well as flavonoids (rutin, luteolin, quercetin, and kameferol) were obtained from Sigma (St. Louis, MO). Deionized water (18 $\Omega$) was prepared using a Milli-Q® purification system (Millipore Corp., New Bedford, MA).

### *Broccoli production*

Two varieties of broccoli (*Brassica oleracea*)—Majestic and Legacy—were used; Legacy was grown in California under normal production conditions. The Majestic variety was grown and handled as previously described, except that the concentration of sodium selenate solution added varied depending of the desired broccoli Se concentration.[27] The different broccoli samples were given the following code names: 0SM, 5SM, 100SM, 1000SM, CGL100, CGL80, and OGL100, where M = Majestic, L = Legacy, CG = conventionally grown, OG = organically grown, and S = Se; the number in front indicates the Se concentration of varieties fertilized with Se, while the number following the symbol refers to the percentage of transpired water that was replaced by irrigation (see below).

### *Legacy production: organic versus conventional*

Broccoli variety Legacy was grown in two field sites in central California; one site was certified for organic farming (4.0 ha at Harris Farms, Five Points, CA; soil classified as a silty clay loam), and the other site was used for conventional farming (20 ha at Harris Farms; soil classified as silty clay loam). Broccoli was planted by seeding. Water was applied by a sprinkler system for about 30 days after seeding and then followed by surface drip irrigation (T-tape) for the remainder of the season. Irrigation was based in part on evapotranspiration data provided by a local weather station; water was supplied as 100% or 80% of evapotranspiration losses. Broccoli plants were harvested from three 1-$m^2$ areas within each respective treatment, and treatments were replicated at least four times. Whole plants were separated into leaf, stem, and floret, prepared, and analyzed as described later.

### *Extraction of phenolic components*

Broccoli extracts were prepared identically: 0.5 g of finely ground freeze-dried broccoli powder was covered with 10 mL of 6:4 (vol/vol) methanol/water, sonicated at room temperature for 30 minutes, and then centrifuged. The supernatant was removed, and the process was repeated once. The extracts were combined and evaporated to dryness in a rotary evaporator at room temperature, and the solid residue was redissolved in 1 mL of 6:4 (vol/vol) methanol/water and filtered (pore size, 0.22 $\mu$m; polyvinylidenedifluoride). HPLC and liquid chromatography (LC)/mass spectrometry (MS) analyses were performed on these samples.

### *LC/ultraviolet (UV)/MS conditions*

The analyses were performed on an Agilent Technologies (Palo Alto, CA) LC/MSD SL HPLC apparatus (model 1100) equipped with a quaternary pump (G1311A), an autosam-

pler (G1313A), photodiode array (G1315B), degasser (G1379A), and column heater (G1316A) and controlled by the Agilent software, HPCore Chemstation. Separation of the main phenolic components of broccoli extracts was achieved with a Waters Chromatography (Milford, MA) Symmetry column (C18; particle size, 5 μm; 3.9 i.d. × 150 mm) with a sentry-guard column (C18; particle size, 5 μm; 3.9 i.d. × 20 mm). The mobile phase was a mixture of water–formic acid (A = 0.1% formic acid in water) and acetonitrile–formic acid (B = 0.1% formic acid in acetonitrile). The mobile phase began with a gradient of 5–20% B in 25 minutes, was held at 20% B until 35 minutes, then increased to 30% B until 58 minutes, and finally brought to 100% B to wash the column for 5 minutes. The injection volume was set at 60 μL. The flow rate was 0.7 mL/min, and the column temperature was set to 25°C. Chromatograms were recorded at 350, 330, and 310 nm for peak intensities, and UV spectra were recorded from 200 to 550 nm. The LC system was directly connected with the MSD detector without stream splitting. The mass spectrometer was equipped with an electrospray ionization (ESI) source that was operated in negative ion mode. Mass spectral data were collected in both full scan and single ion monitoring modes (ions chosen are described below). Fragmentor voltage was held constant for all runs at 100 V. Conditions included a drying gas flow of 13 L/min, drying gas temperature of 350°C, and nebulizer pressure of 50 psi.

Identifications of common flavonoids and other phenolic compounds were made on ESI spectra obtained from this instrument. Some of the identifications were confirmed with selective reaction monitoring (SRM, or $MS^2$) and continuous reaction monitoring (CRM, or $MS^3$) experiments, which were performed on a Hewlett Packard (Palo Alto) model 1100 HPLC apparatus that was interfaced to an ion trap mass spectrometer (LCQ mass spectrometer, Finnigan, San Jose, CA) equipped with an ESI source. The column in this case was a Waters Symmetry column (2.1 × 150 mm, 3.5 μm) with entry guard column (C18; particle size, 5 μm; 3.9 i.d. × 20 mm). The mobile phase contained the same solvents as described above; however, the linear gradient was 15–35% B in 35 minutes and then brought up to 100% to wash the column. The flow rate was 0.2 mL/minute, and injection volume was 15 μL.

### *Acidic hydrolysis: confirmation of flavonoid derivatives*

Some of the identifications of glycosylated flavonoids were confirmed by the analysis of the aglycones obtained from the hydrolysis of the extract and then comparing with commercially available standards. Hydrolysis conditions consisted of treating 0.5 mL of the extract with 0.1 mL of concentrated HCl and then heating the mixture to 95°C for 1.5 hours.

### *Basic hydrolysis: confirmation of phenolic acid derivatives*

To liberate the phenolic acids, saponification of the broccoli samples was performed. A solid broccoli sample (0.200–0.250 g) was covered with 5 mL of a basic solution (2 *N* NaOH, 10 m*M* EDTA, and 1% ascorbic acid). The reaction mixture was stirred with heating (at 30°C) for 30 minutes.[28] The reaction was quenched and adjusted to pH 3 via the addition of 8 *N* HCl. Work-up consisted of extraction with ethyl acetate (2 × 5-mL portions) using sonication for 20 minutes. Nitrogen was used to evaporate the organic solvent. The solid residue was dissolved in 2 mL of 50:50 (vol/vol) methanol/water and filtered (particle size, 0.22 μm; polyvinylidene fluoride) prior to HPLC analysis. An HPLC/UV method developed for the detection and quantitation of phenolic acids in foods that has been described elsewhere[29] was used for the analysis of the freed phenolic acids. Mobile phase consisted of solvent A = 0.1% formic acid in deonized water; solvent B = methanol. Linear gradient was 5–30% B in 50 minutes and then hold at 30% for 15 minutes. The column was a Phenomenex (Torrance, CA) Luna C18-high purity silica (150 × 4.6 mm), the flow rate was set at 0.7 mL/min, and temperature was 25°C. Monitoring was at 270 and 325 nm. MS data on the saponified samples were collected with this same mobile phase using both full scan and single ion monitoring mode.

### *SF analysis*

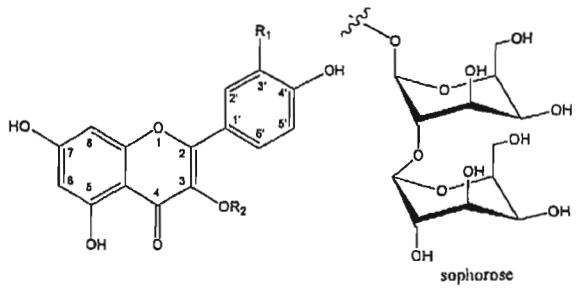
Analysis of SF was by the method described by Matusheski *et al.*[24] In brief, 1 part of finely ground freeze-dried broccoli was mixed with 9 parts of deionized water, wrapped in foil to avoid light exposure, and hydrolyzed for 8 hours at room temperature. The broccoli slurry was filtered through cheesecloth, and an internal standard was added (benzyl isothiocyanate) before 0.5 mL of broccoli extract was extracted with 1.0 mL of acetonitrile. SF concentration was determined in the acetonitrile extract by gas chromatography. The original broccoli concentration of SF was calculated using an SF standard curve, and the data were corrected for extraction efficiency by using the internal standard.

### *Statistical analysis*

Significant differences in compound content were determined using analysis of variance (one-way). Where a significant effect ($P \leq .05$) was found, Tukey's Studentized range test was used to determine differences between means.

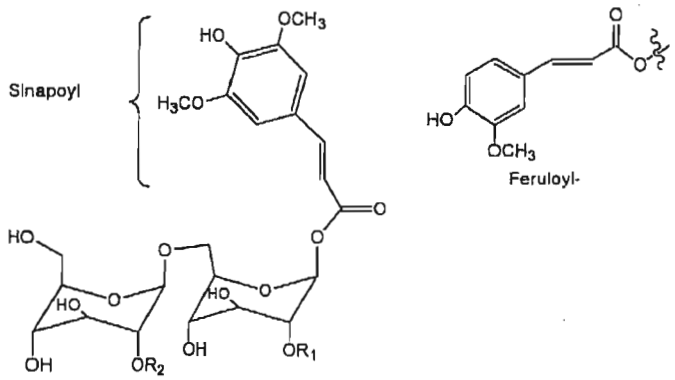
## RESULTS AND DISCUSSION

It is has been estimated that 40% of all human cancers may be a result of poor diets. Some of the relationship between diet and cancer is because of ingestion of potential carcinogens through food, but the greatest impact of diet on cancer is by consumption (or lack of consumption) of compounds that prevent cellular damage and thus reduce the incidence of cancer.[30] Many studies report an especially strong inverse relationship between the intake of cruciferous vegetables and the risk for many cancers,[31,32] an association that is stronger than the association between cancer risk and fruit and vegetable intake in general.[33] Verhoeven *et al.*[34]




| Compound | $R_1$ | $R_2$ | MW |
|---|---|---|---|
| Quercetin-3-O-sophoroside (**5**) | OH | sophorose | 626 |
| Kaempferol-3-O-sophoroside (**4/6**) | H | sophorose | 610 |
| Isomer of kaempferol-3-O-sophoroside (**4/6**) | | | 610 |

A)




B)

| Compound | $R_1$ | $R_2$ | MW |
|---|---|---|---|
| 1,2-disinapoyl gentiobiose (**7**) | sinapoyl | H | 754 |
| 1-sinapoyl-2-feruloyl gentiobiose (**8**) | feruloyl | H | 724 |
| 1,2-diferuloyl gentiobiose (**9**) | feruloyl | H | 694 |
| 1,2,2'-trisinapoyl gentiobiose (**10**) | sinapoyl | sinapoyl | 960 |
| 1,2'-disinapoyl-2-feruloyl gentiobiose (**11**) | feruloyl | sinapoyl | 930 |

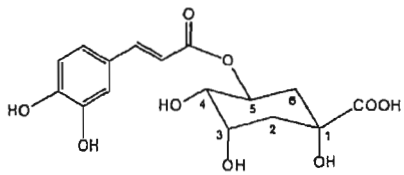



**FIG. 1.** Structure of the phenolic components and derivatives in broccoli. **A:** Glycosylated flavonoids (**4–6**). **B:** Hydroxycinnamic esters (**7–11**). **C:** 5-*O*-Caffeoylquinic acid (**3**), also referred to as chlorogenic acid. 3-*O*-Caffeoylquinic acid (**2**), also referred to as neochlorogenic acid, is not shown; however, substitution would be at position 3.

TABLE 1. RETENTION TIMES, PEAK IDENTITY, AND MS RESULTS FOR BROCCOLI SAMPLE 1000SM

| | *Peak identification* | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Caffeoyl derivatives (chlorogenic acids)* | | | *Flavonoid derivative* | | | *Feruloyl and sinapoyl derivatives* | | | | | |
| | *1* | *2* | *3*[a] | *4* | *5* | *6* | *7* | *8* | *9* | *10* | *11* | *12*[b] |
| Retention time (minutes) | 6.9 | 10.3 | 14.0 | 21.3 | 21.3 | 23.9 | 33.1 | 35.8 | 38.5 | 42.4 | 45.3 | 51.1 |
| [M-H] | 353 | 353 | 353 | 609 | 625 | 609 | 753 | 723 | 693 | 959 | 929 | 849 |
| SRM | 191 | 191 | 191 | 446 | 300 | 429 | 529 | 499 | 499 | 735 | 705 | 714 |
| | | | | 284 | | 285 | 289 | | 477 | | | 687 |
| CRM | | | | 256 | 271 | 256 | | | | | | |

[a]Peak 3 at retention time 14.0 minutes was confirmed to be 5-*O*-caffeol quinic acid via co-injection of commercially available standard.

[b]UV-visible spectrum of this peak contains $\lambda_{max}$ at 240, a peak at 325, and a pre-shoulder at 294, which is consistent with hydroxycinnamic moieties.

reviewed seven cohort studies and 87 case control studies and reported that 67% of the case control studies found inverse associations between total crucifer intake and cancer risk. Broccoli was significantly related to decreased cancer incidence in 56% of the control studies, and cohort studies found a significant inverse association between broccoli intake and risk for all cancers.

The chemoprotective effects of broccoli are likely the result of ingestion of numerous bioactive components, with three of the most important being phenolics, glucosinolates, and (under special conditions) seleno-molecules. Indeed a patented form of broccoli sprouts has been produced with an enhanced concentration of glucosinolates.[35] Thus the potential health benefits of broccoli make it a plant that fits well within the definition of "functional food." However, preliminary evidence led us to believe that increasing the concentration of one compound, especially Se, caused a concomitant decrease in the others; *i.e.*, it was not possible to maximize all the functional characteristics of broccoli simultaneously.

Broccoli is normally not a good source of Se, but when it is grown on Se-rich soil or medium, it will accumulate large amounts. We have previously reported that high-Se broccoli inhibited the formation of chemically induced preneoplastic lesions in rat colon,[27] spontaneous intestinal tumors in mice,[36] and mammary tumors in rats.[37] It was hypothesized that increasing the Se content of broccoli would allow synergistic interaction with other bioactive components, thus increasing its overall chemoprotective benefit. However, in the present report we have characterized glucosinolate and phenolic compounds in broccoli, and have shown that Se fertilization has a major impact on which phenolic compounds predominate, as well as decreases glucosinolate concentrations.

### *Identification of major phenolic components*

An extract of the Majestic broccoli with no Se treatment (0SM) was analyzed by LC/UV/MS and was shown to contain several hydroxycinnamic esters [caffeoyl (**1–3**) and sinapoyl, feruloyl derivatives (**7–11**)] and glycosylated flavonoids [quercetin and kaempferol derivatives (**4–6**)]. Structures of identified peaks are depicted in Figure 1. Analysis of all samples under the same conditions demonstrated that, for the most part, the same compounds with the same mass weight were present in all samples (peak **12** was not detected in two samples). The identities of the peaks were assigned using retention times and LC/MS in full scan mode; further confirmation was achieved by single ion monitoring and examination of the hydrolysis products. Retention times and masses by HPLC-MS are shown in Table 1 (values are for sample 1000SM).

#### *Hydroxycinnamate esters*

Although not directly observable via the LC/UV chromatogram, single ion monitoring confirms three peaks with mass of 353. Compounds **1–3** gave the same [M-1] ion at *m/z* 353 and are isomeric caffeoylquinic acids (also referred to as chlorogenic acids) (Fig. 1C). The base peak in the $MS^2$ spectrum is an ion at *m/z* 191 corresponding to the quinic acid moiety.[38] Although six chlorogenic isomers are known to exist, only two of these isomers have been previously reported in broccoli, 3-*O*-caffeoylquinic (**2**) and 5-*O*-caffeoylquinic acid (**3**).[39] The identity of **1** has not been confirmed. Literature reports indicate the most abundant chlorogenic acid in broccoli is 3-*O*-caffeoylquinic; for that reason we assign the peak at retention time 10.3 minutes to be **2** since the commercially available 5-*O*-caffeoylquinic **3** had a retention time of 14.0 minutes.

The sinapoyl and feruloyl derivatives detected (**7–11**) have been previously reported.[40,41] The base peak for the sinapoyl derivatives (**7**, **8**, **10**, and **11**) indicates loss of fragment of 224 (sinapic acid) except for **9**, where a loss of 194 is observed (loss of ferulic acid). The UV spectrum for peak **12** is indicative of the presence of a hydroxycinnamic moiety, yet the [M-1] ion observed is *m/z* 850 nm, which does not coincide with

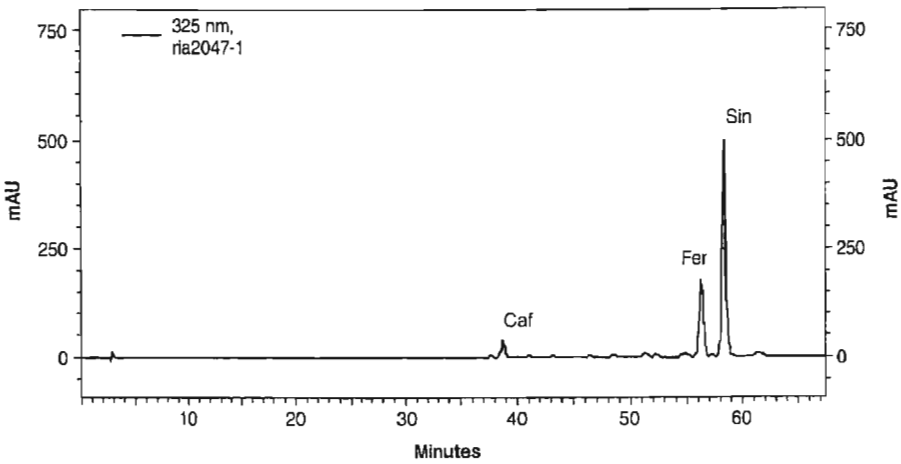



**FIG. 2.** HPLC/UV trace of saponified broccoli samples. Caf, caffeic acid; Fer, ferulic acid; Sin, sinapic acid.

previously reported phenolic components. The $MS^2$ base peak is 687, indicating the loss of 163, consistent with a *p*-coumaric phenolic moiety[38]; however, no *p*-coumaric acid is observed (see below), and the UV spectrum has $\lambda_{max}$ at 220, a pre-shoulder at 295, and a peak at 325 nm, which is consistent with ferulic, sinapic, and caffeic acids (all three have similar UV spectra). The second major peak in the $MS^2$ spectrum is loss of 135, which corresponds to decomposition of caffeic acid. The sugar moieties do not contain any chromophores and are therefore UV silent; however, they can cause the retention times to shift dramatically.

Saponification (basic hydrolysis) was performed to liber-

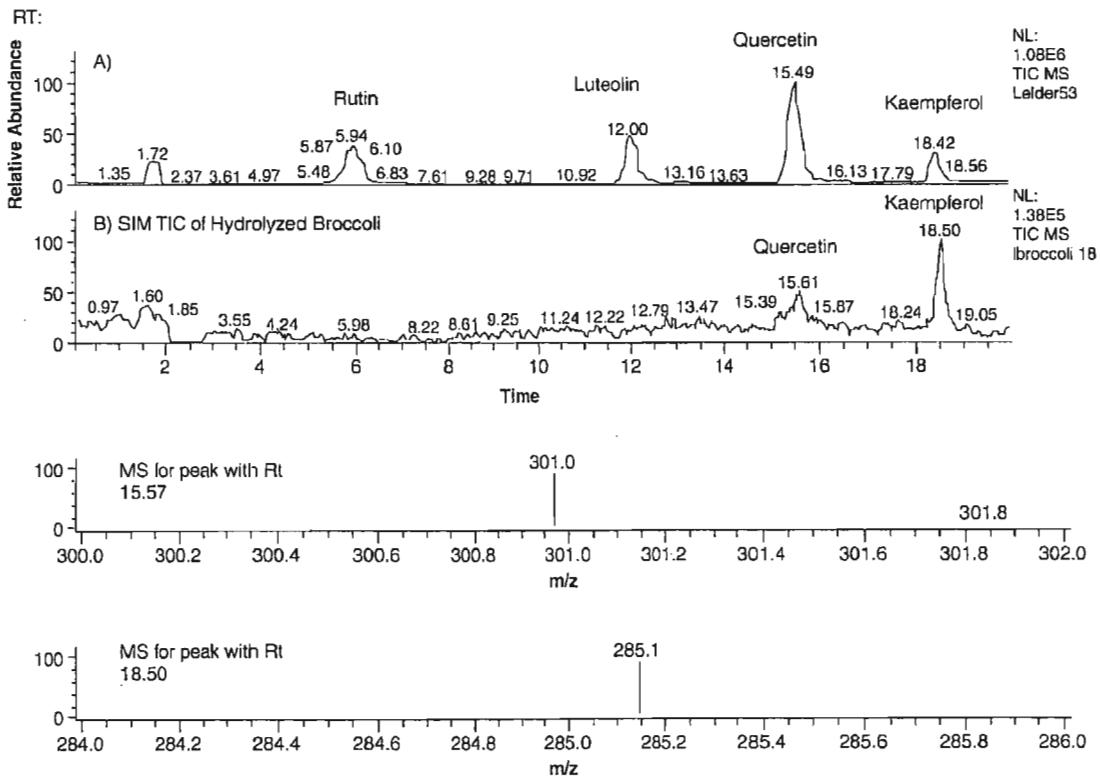



**FIG. 3.** **A:** The total ion chromatograms (TICs) of quercetin, kaempferol, luteolin, and rutin. **B:** Single ion monitoring (SIM) chromatogram and mass spectra of hydrolyzed broccoli sample. Rt, retention time.

TABLE 2. COMPARISONS OF THE PHENOLIC COMPOUNDS PRODUCED IN THE VARIOUS BROCCOLI SAMPLES ANALYZED

| *Broccoli sample* | *Peak identification* | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *1*[a] | *2* | *3* | *4*[b] | *5* | *6* | *7* | *8* | *9* | *10* | *11* | *12* |
| 0 SM | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| 5 SM | 4.3 | 0.9 | 0.6 | 0.4 | 0.6 | 0.4 | 0.9 | 1.1 | 1.2 | 1.3 | 1.9 | 0.2 |
| 100 SM | 1.7 | 1.0 | 3.5 | 0.4 | 0.6 | 0.4 | 0.9 | 0.9 | 0.9 | 1.4 | 2.1 | 0.3 |
| 1000 SM | 7.4 | 0.7 | 0.2 | 0.3 | 0.7 | 0.6 | 0.3 | 0.5 | 0.5 | 0.3 | 0.4 | 0.3 |
| CG L 100 | 3.2 | 0.7 | 3.2 | 1.7 | 0.7 | 0.14 | 1.7 | 1.2 | 0.9 | 1.0 | 0.7 | ND |
| GC L 50 | 2.2 | 0.8 | 2.3 | 1.7 | 0.7 | 0.08 | 2.0 | 1.2 | 1.0 | 0.1 | 0.8 | ND |
| OG L 100 | 2.5 | 0.7 | 3.2 | 1.6 | 0.7 | 1.0 | 1.1 | 0.5 | 0.3 | 1.0 | 0.4 | 0.03 |

The amount of a compound observed in Majestic broccoli with no Se fertilizer (0SM) was arbitrarily set to 1.0 (100%), and changes induced by treatment are presented as a factor of 1.0. ND, not detected.

[a]Areas obtained for chlorogenic acid derivatives **1–3** were obtained by monitoring for the single ion $m/z$ 353 (single ion monitoring).

[b]Areas obtained for each $m/z$ 609 and 625 were obtained using single ion monitoring at retention time 21.3 minutes.

ate the monomeric phenolics, since *cis–trans* isomerization, decomposition, and transesterification can occur under acidic conditions. Three hydroxycinnamic acids—caffeic, ferulic, and sinapic—were observed (Fig. 2); identities of these peaks were confirmed by spiking of the saponified solution with commercially available standards as well as comparison of UV spectra. No *p*-coumaric acid was observed, indicating that peak **12** most likely does not contain a *p*-coumaroyl substituent. Analysis of the unhydrolyzed extract found no evidence of free phenolic acids.

### *Glycosylated flavonoids*

Two components co-eluted (retention time = 21.3 minutes), and the overlapped peaks gave $m/z$ 608.9/625.0, which are consistent with reported kaempferol-3-*O*-sophoroside and quercetin-3-*O*-sophoroside, respectively.[41] A third peak with isomeric $m/z$ 609.0 eluted at retention time 23.9 minutes. Acid hydrolysis of the broccoli extract was further analyzed by LC/MS/MS for presence of aglycones. Compared with commercial standards, retention times and masses of the detected products ([M-1]) were consistent with quercetin ($m/z$ 301) and kaempferol ($m/z$ 285) (Fig. 3). The aglycone luteolin was also included in the commercially available mixture since it has the same molecular mass (286) as kaempferol, but was not detected in the hydrosylate. These results are consistent with kaempferol as the flavonoid backbone for the peaks with $m/z$ of 609. We cannot assign the exact structure to peaks labeled **4** and **6**; however, evidence (retention time and the mass data) indicates that they are likely to be isomers with the difference perhaps being the position of the sugar moiety (substitution at position 7 vs. 3 based on previous literature reports indicating possible substitution at that position 7).[42] Analysis of the peak with [M-1] at $m/z$ 625 through SRM (or $MS^2$) gave a major ion at $m/z$ 300.1. CRM (or $MS^3$) gave a product ion spectrum similar to that of the quercetin standard, suggesting the peak at retention time 21.3 minutes might contain quercetin-3-*O*-sophoroside (**5**).

### *Effect of variety and culture conditions on relative concentrations of the phenolic components identified*

The most prominent differences in phenolic profiles were between broccoli varieties (although variety was confounded with production conditions, so we cannot say with absolute certainty that variety was the sole determinant of differences). Relative to Legacy, the Majestic variety had an

TABLE 3. AMOUNT OF MONOMERIC PHENOLIC ACIDS AFTER BASIC HYDROLYSIS ON SOLID BROCCOLI SAMPLES

| *Broccoli sample* | *Phenolic acid (mg/100 g)* | | |
|---|---|---|---|
| | *Caffeic* | *Sinapic* | *Ferulic* |
| CGL100 ($n$ = 3) | 2.1 ± 0.2 (9.6%) | 50.8 ± 4.7 (9.4%) | 16.3 ± 1.3 (8.0%) |
| CGL50 ($n$ = 3) | 4.0 ± 0.2 (6.6%) | 54.4 ± 1.6 (3.1%) | 16.0 ± 1.3 (3.2%) |
| OGL100 ($n$ = 3) | 3.8 ± 0.4 (0.9%) | 40.5 ± 0.4 (0.9%) | 8.5 ± 0.02 (0.3%) |
| 0SM ($n$ = 3) | 9.6 ± 0.1 (1.3%) | 63.7 ± 1.0 (1.7%) | 27.3 ± 0.4 (1.3%) |
| 5SM ($n$ = 3) | 18.9 ± 0.6 (3.4%) | 77.2 ± 1.7 (2.3%) | 43.6 ± 0.9 (2.2%) |
| 100SM ($n$ = 3) | 22.2 ± 0.2 (1.1%) | 87.7 ± 2.0 (2.2%) | 46.5 ± 0.2 (1.7%) |
| 1000SM ($n$ = 7) | 3.7 ± 0.2 (5.9%) | 28.7 ± 1.8 (6.6%) | 31.5 ± 2.0 (6.3%) |

Amounts are mean values reported in milligrams/100 g of sample. Coefficients of variation are in parentheses.

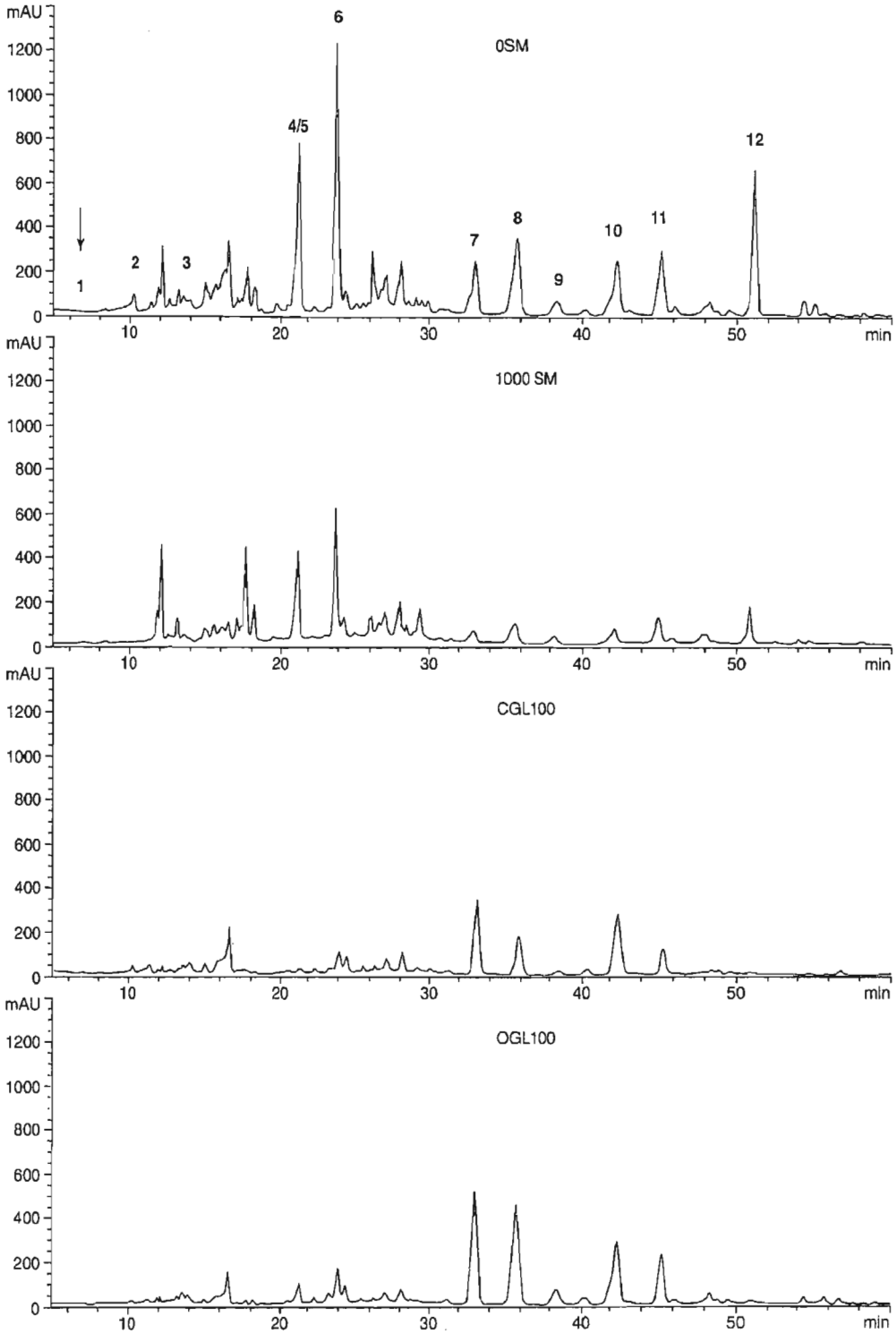



**FIG. 4.** HPLC/UV trace for four methanol/water broccoli extracts (0SM, 1000SM, CGL100, and OGL100) monitored at 350 nm. Phenolic components **1–12** are labeled. Although peak **1** is not detected via UV, it was detected with MS (see text).

abundance of the flavonoids quercetin and kaempferol, and feruloyl and sinapoyl esters were greatly reduced. Peak **12** was also abundant in Majestic broccoli, but nearly undetected in the Legacy broccoli.

Comparisons within the Majestic variety showed that Se fertilization did not have much effect on which phenolic compounds were produced, but it had a great effect on the total amount produced. To facilitate comparison between treatments, the amount of a compound observed in Majestic with no Se fertilizer was arbitrarily set to 1.0, and changes induced by treatment are presented in Table 2 as a factor of 1.0. Overall, fewer phenolic compounds were produced in plants with Se concentrations of 1,000 $\mu$g of Se/g of dry weight. However, four treatments were examined with Se contents of 0, 5, 100, and 1,000 $\mu$g of Se/g of dry tissue, and Se fertilization up to 100 $\mu$g of Se/g of dry tissue actually increased or had minimal to no effect on production of eight of 12 identified compounds. However, when fertilization was increased and the plant contained 1,000 $\mu$g of Se/g of dry tissue, 11 of 12 identified compounds decreased. Total phenolics were measured on these four samples using the standard Folin Ciocalteu colorimetric assay. Sample 0SM yielded 6.78 mg of gallic acid equivalents/g of broccoli. The values obtained for 5SM, 100SM, and 1000SM were 9.15, 6.76, and 4.31 mg of gallic acid equivalents/g of broccoli, respectively. Results confirmed the decrease in absorbance observed by LC/UV analyses. Total phenolics in three classes increased with Se fertilization up to 100 $\mu$g of Se/g of dry tissue, but dropped dramatically with a further increase to 1,000 $\mu$g of Se/g of dry tissue. Determination of liberated caffeic, ferulic, and sinapic acids in the broccoli also indicated the drop in these components for the 1000SM broccoli sample (Table 3).

There may be evidence for a physiological breaking point between 100 and 1,000 ppm of Se, as Bird *et al.*[43] reported that garlic with an Se concentration of 1,355 $\mu$g/g of dry weight contained primarily Se-methylselenocysteine (SeMSC) as the Se species, whereas garlic with an Se concentration of 296 $\mu$g/g contained primarily $\gamma$-glutamyl SeMSC. Thus it may be that a certain point the Se concentration in a plant exceeds the ability of the plant to completely detoxify it, resulting in a toxic stress and depressed production of metabolites such as phenolic components.

Within the Legacy variety, organic and conventional farming practices can be compared directly within the 100% water treatments. There were very few visually apparent differences (Fig. 4), and comparison of the relative abundances of each particular compound (Table 2) confirmed that almost all of the same compounds were found at the same abundances; further, there was no detectable pattern that suggested that water treatment or farming method had a greater influence on phenolic production. Analysis of total caffeic, sinapic, and ferulic acids gave a similar result, *i.e.*, no detectable pattern of production method or water treatment having a greater effect on phenolic production (Table 3).

### *Effect of selenium on glucosinolates, SF, and Se concentration on broccoli*

Se fertilization also affected production of another class of secondary plant compounds, the glucosinolates. In general, increased Se fertilization of plants resulted in a dose-

TABLE 4. TOTAL GLUCOSINOLATES (GS), INDOLE GLUCOSINOLATES (INDOLE GS), ALIPHATIC GLUCOSINOLATES (ALIPHATIC GS), GLUCORAPHANIN (GP), SF, AND SE CONTENT OF FREEZE-DRIED BROCCOLI SAMPLES OR AQUEOUS EXTRACTS OF BROCCOLI, BROCCOLI GROWN WITH DIFFERENT CONCENTRATIONS OF SE FERTILIZER, OR FIELD-GROWN WITH CONVENTIONAL OR CERTIFIED ORGANIC TECHNIQUES

| *Freeze-dried broccoli* | *Total GS* | *Indole GS* | *Aliphatic GS* | *GP* | *Total content of lyophilized broccoli* | | *Content of lyophilized aqueous extract ($\mu M$)* | |
|---|---|---|---|---|---|---|---|---|
| | | | | | *SF* | *Se* | SF | Se |
| Se treatment | | | | | | | | |
| 0 ppm Se | 15.9 ± 1.7$^{a}$ | 2.4 ± 0.6 | 11.4 ± 1.4$^{a}$ | 7.0 ± 0.5$^{a}$ | | 0.4 ± 0.01$^{a}$ | 239 ± 2$^{a}$ | 1.3 ± 0.2 |
| 100 ppm Se | 14.0 ± 2.4$^{a,b}$ | 2.5 ± 0.8 | 8.0 ± 1.8$^{a,b}$ | 6.5 ± 1.1$^{a}$ | | 98.6 ± 3.7$^{b}$ | 157 ± 3$^{b}$ | 35.4 ± 1.3 |
| 10,000 ppm Se | 11.6 ± 1.8$^{b}$ | 2.5 ± 0.2 | 6.5 ± 1.7$^{b}$ | 4.4 ± 0.5$^{b}$ | | 879.2 ± 3.2$^{c}$ | 41 ± 3$^{c}$ | 354 ± 5.6 |
| Organic and conventional production | | | | | | | | |
| Conventionally grown, 80% $H_2O$ | 16.5 ± 0.9 | 7.2 ± 1.0$^{b}$ | 6.1 ± 0.9 | 4.9 ± 0.8 | 3.9 ± 0.1$^{a}$ | 0.14 ± 0.01 | | |
| Conventionally grown, 100% $H_2O$ | 20.0 ± 3.2 | 9.3 ± 1.4$^{a}$ | 8.1 ± 1.8 | 6.2 ± 1.4 | 4.0 ± 0.1$^{a}$ | 0.12 ± 0.01 | | |
| Organic grown, 100% $H_2O$ | 17.6 ± 1.0 | 7.0 ± 1.0$^{b}$ | 8.0 ± 0.8 | 6.9 ± 0.8 | 1.6 ± 0.1$^{b}$ | 0.05 ± 0.002 | | |

Data are mean ± standard error values and are in units of micromoles/gram of dry weight except as noted for the lyophilized aqueous extract of broccoli [20:1 (vol/vol) water/broccoli] ($n$ = 4 for Se treatment; $n$ = 3 for organic vs. conventionally grown). Means in the same column with different superscripts are significantly different. Se treatment and organic versus conventional production were analyzed separately.

dependent decrease in all classes of glucosinolates. Glucoraphanin, the parent compound of SF, decreased from 8.7 to 5.1 μmol/g of dry weight (41% decreased) in high-Se broccoli compared with 8.7 μmol/g of dry weight in low-Se broccoli (Table 4); this resulted in a corresponding decrease in SF from 239 to 41 μM (82% decrease). Aliphatic glucosinolates showed a more modest decline, from 12.6 to 8.3 μmol/g, a 35% decrease (Table 4).

Production method affected glucosinolate content, as both water stress and organic farming decreased glucosinolate content relative to unstressed conventionally grown broccoli. Interestingly, water stress and organic production gave similar results for most compounds. These results seem contrary to what would be expected as phenolic compounds have been considered to increase in times of stress.[10] Numbers in the present report are limited, and certainly results cannot be generalized, but neither phenolic acid nor glucosinolate data are suggestive of greater concentrations of bioactive compounds in organically grown broccoli.

Overall these data illustrate the variability of bioactive compounds. Further, they show that attempts to maximize a certain bioactive compound may result in a decrease in the content of another compound. If the agricultural and food industry wishes to promote a product based on "functional" properties, the compound of interest must be characterized, and sources of variation must be determined. Foods are not produced with the precision of pharmaceuticals, and the prospect of significant variation is always a potential, if not a reality.

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